Comprehensive assessment of tumour budding by cytokeratin staining in colorectal cancer

Rieger, Gregor ; Koelzer, Viktor H ; Dawson, Heather E ; Berger, Martin D ; Hädrich, Marion ; Inderbitzin, Daniel ; Lugli, Alessandro ; Zlobec, Inti

Abstract: Aims Tumour budding in colorectal cancer (CRC) is a recognized prognostic parameter. The aim of this study was to address the use of cytokeratin immunostaining for the visualization and scoring of tumour buds. Methods and results Ten hotspots (0.238 mm2) of peritumoural budding (PTB) and intratumoural budding (ITB) were evaluated in surgical resections from 215 patients. The budding counts in the 10 densest regions anywhere in the tumour were combined into an overall tumour budding (OTB) score. The PTB, ITB and OTB hotspot with the maximum budding count was then evaluated. Finally, continuous and cut-off values of 10 buds per high-power field (HPF) (PTB10HPF), five buds per HPF (ITB10HPF) and eight buds per HPF (OTB10HPF) were used to categorize budding counts into low-grade and high-grade scores. All budding scores were highly correlated. PTB and ITB counts were associated with many clinicopathological features, including tumour stage, lymph node and distant metastasis, venous and lymphovascular invasion, and disease-free survival (DFS) (all $P < 0.05$). Analyses of OTB counts recapitulated these associations, including a lower DFS with a greater number of tumour buds ($P = 0.0309$; hazard ratio 1.0332, 95% confidence interval 1.003–1.062). One OTB hotspot performed similarly to 10 OTB hotspots in terms of relationship with outcome. These statistical significances were largely lost when cut-offs were applied to PTB, ITB or OTB counts. Conclusions An OTB count in a single hotspot on cytokeratin-stained CRC tissue sections is a fast and reliable prognostic scoring system for the assessment of tumour budding. This approach should be considered in future studies.

DOI: https://doi.org/10.1111/his.13164

DR VIKTOR HENDRIK KOELZER (Orcid ID : 0000-0001-9206-4885)

Received Date : 07-Dec-2016

Revised Date : 03-Jan-2017

Accepted Date : 05-Jan-2017

Article type : Original Article

Comprehensive assessment of tumour budding on cytokeratin stains in colorectal cancer.

Gregor Rieger*[a], Viktor H. Koelzer*[a,b], Heather E. Dawson[a], Martin D. Berger[c,d], Marion Hädrich[e], Daniel Inderbitzin[e,f], Alessandro Lugli[a], Inti Zlobec[a]

a Institute of Pathology, University of Bern, Murtenstrasse 31, Bern, 3010, Switzerland

b Institute of Pathology, Cantonal Hospital Baselland, Liestal, Mühlemattstrasse 11, 4410, Switzerland

c Department of Medical Oncology, Bern University Hospital, Switzerland

d Division of Medical Oncology, Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

e Departments of Visceral Surgery and Medicine, Bern University Hospital, Bern, Switzerland

f Department of Surgery, Bürgerspital Solothurn, Solothurn, Switzerland

** Equally contributing first authors*

Short title: Tumour budding in colorectal cancer

Corresponding author
Prof. Inti Zlobec, PhD
Institute of Pathology, University of Bern
Murtenstr. 31, CH-3010 Bern, Switzerland
Telephone: 0041-31-632-8755
Fax: 0041-31-632-4995
E-mail: inti.zlobec@pathology.unibe.ch

Conflict of interest statement and funding disclosures:

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, royalties or patents received or pending.

ABSTRACT

Background: Tumour budding in colorectal cancer (CRC) is a recognized prognostic parameter. Aim of this study is to address the use of cytokeratin immunostaining for visualization and scoring of tumour buds.

Methods: Ten hotspots (0.238 mm^2) of peritumoural (PTB) and intratumoural (ITB) budding were evaluated in surgical resections from 215 patients. The budding counts in the 10 densest regions anywhere in the tumour were combined into an overall tumour budding (OTB) score. The PTB, ITB and OTB hotspot with the maximum budding count was then evaluated. Finally, continuous and cut-off values of 10 buds/HPF (PTB_{10HPF}), 5 buds/HPF (ITB_{10HPF}) and 8 buds/HPF (OTB_{10HPF}) were used to categorize budding counts into low/high-grade scores.

Results: All budding scores were highly correlated. PTB and ITB counts were associated with many clinicopathological features including tumour stage, lymph node and distant metastasis, venous and lymphovascular invasion and disease-free survival (DFS) (all $p<0.05$). Analyses of OTB counts recapitulated these associations, including a lower DFS with a greater number of tumour buds ($p=0.0309$; HR (95%CI): 1.032 (1.003-1.062)). One OTB hotspot performed similarly as ten OTB hotspots in terms of relationship with outcome. These statistical significances were largely lost when cut-offs were applied to PTB, ITB or OTB counts.

Conclusions: OTB count in a single hotspot on cytokeratin-stained CRC tissue sections is a fast and reliable prognostic scoring system for the assessment of tumour budding. This approach should be considered in future studies.

Keywords: colorectal cancer; prognosis; pathology; precision medicine

INTRODUCTION

Single cancer cell invasion or collective migration of small tumour clusters in the stroma of colorectal carcinoma (CRC) is referred to as tumour budding [1]. The presence of tumour buds at the invasion front (peritumoural budding, PTB) and in the tumour centre (intratumoural budding, ITB) is linked to an aggressive tumour phenotype with adverse clinicopathological features and poorer survival of CRC patients [2-6]. Quantification of tumour buds has major potential for clinical management: First, enumeration of tumour buds may improve the risk stratification of patients with endoscopically resected CRC and can aid the decision for colorectal surgery [6-10]. Second, assessment of tumour budding may allow the identification of high risk stage II CRC patients for intensified follow up and adjuvant therapy trials [2, 4, 11]. Last, detection of tumour buds in pre-operative biopsies may indicate an increased risk of nodal metastases and resistance to neoadjuvant therapy in rectal cancer patients [12, 13].

Recently, an international panel of experts recommended guidelines for the assessment of tumour budding and outlined several areas that would benefit from a higher level of evidence in future studies [14]. In particular, the role of cytokeratin staining, its corresponding scoring system (counts versus low/high-grade categories and number of fields) as well as the location within the tumour (intra- or peri-tumoural budding) were outlined as critical areas for further pursuit.

In this study, we perform a comprehensive methodological assessment of tumour budding on cytokeratin stained sections in order to answer questions regarding: optimal tumour location for scoring, the use of continuous/categorical scoring approaches and the number of fields.

PATIENTS AND METHODS

A sample size calculation was performed in order to determine the number of patients for inclusion in this retrospective cohort. Using lymph node status as outcome, and high-/low-grade budding as predictor, n=160 patients of all stages were required to reach 80% power, and an effect size of OR=2.8. Two-hundred and fifteen non-consecutive primary CRC patients could be included. These patients were treated between 2002 and 2011 at the Bern University Hospital, Switzerland. Histopathological H&E slides were re-reviewed (AL, HD, VHK) according to the UICC TNM 7th edition [15]. Patient characteristics are summarized in **Table S1** and include information on gender, age, histological subtype (adenocarcinoma, mucinous, other), tumour location, pathological tumour (pT) and nodal stage (pN), presence of distant metastasis as determined by clinical and radiographic examination (cM), data on lymphatic (L), venous (V) and perineural invasion (Pn), tumour grade (G), resection status (R), tumour border configuration as percentage of pushing growth pattern (TBC), tumour deposits, pre- and postoperative therapy (Preop Tx; Postop Tx). Microsatellite instability status (MSI) was determined using a panel of three Bethesda markers (BAT25, BAT26 and D2S123) as previously described [16]. Tumours were classified as MSI+ if they had two or more unstable markers and MSI– if all markers were stable. One tumour showing one single unstable marker was not classified. Clinical and survival information was retrieved from patient records. Mean and median follow up were 44 and 32 months, respectively (min and max: 1-142 months). No patients were treated by endoscopic tumour resection or neoadjuvant chemotherapy. Patients were followed up in accordance with the recommendations of the Swiss Society of Gastroenterology for surgically resected colorectal tumours [17]. This includes serial clinical examinations and evaluation of carcinoembryonic antigen (CEA) serum levels, a colonoscopy at 12 and 48 months and yearly computed tomography (CT) scans of the thorax and abdomen for patients with pT3/4 disease following resection. For rectal cancer patients, rectal endosonography or pelvic magnetic resonance imaging (MRI) is performed at 6-monthly intervals for the first 2 years followed by yearly CT scans of

the thorax, abdomen and pelvis. A lower gastrointestinal endoscopy is performed at 6, 18 and 24 months.

Assay methods

Specimens were fixed in 10% neutral buffered formalin. Gross assessment, dissection and sampling was performed in accordance with standard protocols. All diagnostic slides were re-reviewed and the tumour block with the highest budding grade on standard H&E histology was selected for cytokeratin immunohistochemistry (AE1/AE3; Dako, mouse monoclonal, 1:200, enzyme pre-treatment 5 minutes, DAB chromogen; using a Leica Bond III instrument). Double-staining with CD8+ was performed but was not evaluated for this study.

Assessment of tumour budding

Tumour budding was defined as single tumour cells or tumour-cell-clusters of up to 5 cells (≤5 cells) in the tumour stroma of the invasive front (PTB) or within the tumour (ITB) in keeping with previously published definitions [3, 6, 18]. Specifically, tumour budding cells were required to show cytoplasmic positivity and a nucleus in cytokeratin stains. To classify as ITB, tumor buds had to be surrounded by malignant glands on all sides. To classify as PTB, tumor buds had to be localized in the tumor stroma ahead of the invasive front.

Tumour budding was assessed by one observer (GR) trained and supported by a team of expert gastrointestinal pathologists. Slides were first viewed at low-power to identify the densest areas of PTB and ITB. Tumour buds were counted in this area in one HPF (Nikon Eclipse 50i, 40x objective, field diameter 0.55mm, area 0.238mm^2) which was labeled as the hotspot of tumour budding ($PTB_{hotspot}$, $ITB_{hotspot}$). A total of 10HPF in each area were evaluated for PTB_{10HPF} and ITB_{10HPF}. Overall tumour budding scores were defined as the hotspot with the highest score ($OTB_{hotspot}$) and the 10 HPF with the highest budding counts (OTB_{10HPF})**.** The study design is shown in Figure 1. All observers were blinded to clinicopathological data.

Statistics

Disease-free survival (DFS) was defined as the time from surgical resection to recurrence or death, whichever occurred first, with recurrence defined only by the reappearance of the primary CRC. Descriptive statistics were performed for all budding counts. Pearson's correlation coefficient was used to determine the strength of the linear relationship (r). The association of tumour budding as a continuous variable with categorical endpoints was analysed with the Wilcoxon Rank Sum Test and with logistic regression. Previously published cut-offs of 10 buds for the PTB_{10HPF} [19] and 5 buds for the ITB_{10HPF} [20] method were used. For OTB, receiver operating characteristic (ROC) derived thresholds were investigated using death as an endpoint. The Kaplan-Meier method was used to represent survival curves and the log-rank test was used to test significant survival time differences. The Chi-Square or Fisher's Exact tests were used where appropriate. Analyses were performed using SPSS (Version 21) and with SAS (Version 9.4 SAS Institute, Cary, NC). P-values <0.05 were considered statistically significant.

Ethics approval

The use of patient material was approved by the ethics commission of the canton of Bern (KEK-200/14).

RESULTS

Tumour budding was assessed in 1 hotspot or in 10 hotspots containing the densest regions of tumour buds in three different locations: ITB, PTB, or independently of location using an OTB count (Figure 2).

Topographic assessment of tumour budding in CRC

Descriptive statistics for PTB, ITB and OTB are found in **Table 1**. The mean number of PTB counts across 10 HPFs was 8.5 in comparison to 7.6 and 8.0 for ITB and OTB. For the single hotspot, OTB

counts were largest with 15.8 buds/HPF, followed by PTB (13.8 buds/HPF) and ITB (11.8 buds/HPF). All values of PTB, ITB and OTB correlated significantly with each other (**Table 2**).

Tumour budding and association with clinicopathological features

Continuous scores

Tumour budding counts in any location were not correlated with gender, age, resection margin status or MSI status. **Table 3** highlights the associations between PTB, ITB and OTB and other clinicopathological features. Tumour budding counts were significantly associated ($p<0.05$, all) with more advanced T-stage, presence of nodal metastasis, lymphatic invasion, venous invasion, tumour grade, perineural invasion and infiltrating tumour border configuration independent of the location of assessment (PTB, ITB or OTB, hotspot or 10HPF).

DFS times were available for 208 patients with 38 patients relapsing during clinical follow-up. Significant associations of tumour budding counts with a shorter DFS as assessed by PTB_{10HPF} (p=0.0315; HR (95%CI): 1.028 (1.002-1.053)), $ITB_{hotspot}$ (p=0.0126; HR (95%CI): 1.028 (1.006-1.051)), OTB_{10HPF} (p=0.0309; HR (95%CI): 1.032 (1.003-1.062)) and $OTB_{hotspot}$ (p=0.05; HR (95%CI): 1.021 (1.0-1.042)) were identified.

Cut-off scores

In order to determine the impact of cut-off scores on the association of budding with clinicopathological features, we applied a threshold of 10 buds for PTB counts[19], 5 buds for ITB counts [20] and newly identified thresholds of 8 and 14 for OTB_{10HPF} and $OTB_{hotspot}$. Frequencies of high-grade budding for PTB, ITB and OTB are found in **Table S2.**

Table 3 highlights loss of associations when budding scores are dichotomized. Only presence of nodal metastasis and infiltrating tumour border configuration (all $p<0.05$) were reliably predicted by classification according to cut-off scores. Inconsistent associations were also identified between high grade budding cancers and clinical evidence of distant metastasis. Importantly, no associations of

tumour budding as assessed by cut-off scores with DFS were identified (PTB_{10HPF} p=0.6681; ITB_{10HPF} p=0.7198, OTB_{10HPF} p=0.7831, $PTB_{hotspot}$ p=0.8864, $ITB_{hotspot}$ p=0.5243, $OTB_{hotspot}$ p=0.4534).

DISCUSSION

The International Tumour Budding Consensus Conference (ITBCC) in April 2016 gathered experts from around the world to discuss the issues related to tumour budding [14]. One major area of interest was the use of cytokeratin stains for scoring. It was outlined that the evidence supporting the implementation of cytokeratin staining was moderate to low and was an area requiring further work in future studies. We undertook this study to confirm the utility of cytokeratin staining for the evaluation of tumour budding.

Evidence from the literature suggests that both PTB in surgical resections and ITB in preoperative biopsies is not only predictive of lymph node and distant metastasis but also of poorer overall and disease-free survival [3, 6]. However, what qualifies as PTB and ITB in resection specimens is not always clear and borders between PTB and ITB are often blurred. In a first step, we scored ten clearly defined regions of PTB and ITB which were then used to calculate an overall tumour budding score (OTB) to determine the impact of the location of scoring on clinicopathological endpoints. All values of budding are highly correlated with each other. Moreover, we show that the associations identified from PTB and ITB are recapitulated in OTB, suggesting that as long as the densest region of tumour budding is identified, the location (PTB or ITB) does not actually play a role.

Scoring systems for tumour budding include both subjective and more quantitative methods, and often only a single field of view is considered [5, 21, 22]. Previous studies on PTB demonstrate that the evaluation of 1 or 10 hotspots on cytokeratin produces nearly identical results in terms of inter-observer agreement, although with a slight advantage for PTB counted in multiple regions [23]. In this study, we show that the evaluation of a single OTB hotspot performs similarly to the evaluation of 10 OTB regions, suggesting that only one densest region of tumour budding scored anywhere

throughout the tumour would be sufficient. Several arbitrary cut-offs ranging from single cells to small clusters of ≤4 or ≤5 have been used for the definition of a single tumour bud (see [3, 18] for critical discussion). A consistent adverse prognostic impact of tumour budding has been observed in studies using either value [18]. However, a systematic comparison is so far lacking in the literature and needs to be addressed in future investigations.

Another central issue is the use of tumour budding counts or a low/high-grade scoring approach, determined around a cut-off [3, 6]. The distribution of tumour budding counts points to no obvious cut-off that would separate this cohort of patients. Additionally, the ROC curve for tumour budding and lymph node metastasis (or any other endpoint with clinical relevance) presents no evident cut-off point to best discriminate between outcomes. Several more arguments supporting a continuous count of tumour buds can be made: a larger number of tumour buds can potentially have a clinically relevant meaning- we have previously shown this in the context of intratumoural budding in rectal tumour biopsies with the aim of predicting lymph node and distant metastasis [20] Tumors with 9 or 11 buds cannot be so biologically different that they warrant being placed into low and high-grade categories using a cut-off of 10 buds. In this study, we show that the associations of tumour budding with a range of different clinicopathological features, and most importantly with DFS, are only achieved with continuous scores. Based on these arguments, we reason that the number of tumour buds on cytokeratin stains should be recorded, even if these counts are used in a categorical scoring system in a second step.

This study specifically focuses on cytokeratin staining for the evaluation of tumour budding cells. We used AE1/AE3 cytokeratin staining based on previous studies [19, 20, 24]. AE1/AE3 is a keratin cocktail that detects cytokeratin 1-6, 8, 10, 14-16 and 19, but does not detect CK17 or CK18 [25]. The choice of a broad-spectrum anti-cytokeratin is of central importance, as some molecular subgroups of CRC may express a differential cytokine profile. In particular, MSI+ CRC may show a loss of cytokeratin 20

and aberrant expression of cytokeratin 7 [26]. Further, some CRC with neuroendocrine differentiation may show only a focal or dotlike expression of cytokeratin at the invasive front, making a careful interpretation mandatory [27].

To conclude, this study demonstrates that an OTB count in a single hotspot on cytokeratin-stained CRC tissue sections excels in the assessment of tumour budding. Although further studies are needed to validate these findings, a count of OTB encompasses the positive aspects of PTB and ITB and therefore will be applicable to both surgical resections and preoperative biopsies.

Acknowledgments: GR contributed to the study concept and design, data acquisition and preparation of the manuscript. VHK contributed to the study concept and design, data acquisition and interpretation and preparation of the manuscript. HD contributed to the study design, data interpretation and preparation of the manuscript. MDB contributed to data acquisition, quality control of data and critically reviewed the manuscript. MH contributed to data acquisition, quality control of data and critically reviewed the manuscript. DI contributed to data acquisition, quality control of data and critically reviewed the manuscript. AL contributed to the study concept and design, and critically reviewed the manuscript. IZ contributed to the study concept and design, contributed to data acquisition and interpretation, performed statistical analysis and contributed to preparation of the manuscript. The authors thank Ian Tomlinson and Enric Domingo, Wellcome Trust Centre for Human Genetics, Oxford for excellent collaboration and material transfer in analysis of microsatellite instability status. Further, we would like to acknowledge Dr. Irene Centeno, Dr. José Galván and Caroline Hammer from the Translational Research Unit, Institute of Pathology, University of Bern for excellent technical support. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

REFERENCES

1. Prall F. Tumour budding in colorectal carcinoma. *Histopathology* 2007;**50**;151-162.
2. Wang LM, Kevans D, Mulcahy H *et al.* Tumor budding is a strong and reproducible prognostic marker in t3n0 colorectal cancer. *The American journal of surgical pathology* 2009;**33**;134-141.

3. Mitrovic B, Schaeffer DF, Riddell RH, Kirsch R. Tumor budding in colorectal carcinoma: Time to take notice. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 2012;**25**;1315-1325.
4. Nakamura T, Mitomi H, Kanazawa H, Ohkura Y, Watanabe M. Tumor budding as an index to identify high-risk patients with stage ii colon cancer. *Diseases of the colon and rectum* 2008;**51**;568-572.
5. Ueno H, Murphy J, Jass JR, Mochizuki H, Talbot IC. Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. *Histopathology* 2002;**40**;127-132.
6. Koelzer VH, Zlobec I, Lugli A. Tumor budding in colorectal cancer--ready for diagnostic practice? *Human pathology* 2016;**47**;4-19.
7. Beaton C, Twine CP, Williams GL, Radcliffe AG. Systematic review and meta-analysis of histopathological factors influencing the risk of lymph node metastasis in early colorectal cancer. *Colorectal disease : the official journal of the Association of Coloproctology of Great Britain and Ireland* 2013;**15**;788-797.
8. Choi DH, Sohn DK, Chang HJ, Lim SB, Choi HS, Jeong SY. Indications for subsequent surgery after endoscopic resection of submucosally invasive colorectal carcinomas: A prospective cohort study. *Diseases of the colon and rectum* 2009;**52**;438-445.
9. Nakadoi K, Tanaka S, Kanao H *et al.* Management of t1 colorectal carcinoma with special reference to criteria for curative endoscopic resection. *Journal of gastroenterology and hepatology* 2012;**27**;1057-1062.
10. Tateishi Y, Nakanishi Y, Taniguchi H, Shimoda T, Umemura S. Pathological prognostic factors predicting lymph node metastasis in submucosal invasive (t1) colorectal carcinoma. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 2010;**23**;1068-1072.
11. Kevans D, Wang LM, Sheahan K *et al.* Epithelial-mesenchymal transition (emt) protein expression in a cohort of stage ii colorectal cancer patients with characterized tumor budding and mismatch repair protein status. *International journal of surgical pathology* 2011;**19**;751-760.
12. Rogers AC, Gibbons D, Hanly AM *et al.* Prognostic significance of tumor budding in rectal cancer biopsies before neoadjuvant therapy. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 2014;**27**;156-162.
13. Morodomi T, Isomoto H, Shirouzu K, Kakegawa K, Irie K, Morimatsu M. An index for estimating the probability of lymph node metastasis in rectal cancers. Lymph node metastasis and the histopathology of actively invasive regions of cancer. *Cancer* 1989;**63**;539-543.
14. A. Lugli RK, Y. Ajioka, F. Bosman, G. Cathomas, H. Dawson, H. El Zimaity, J.-F. Fléjou, T. P. Hansen, S. Kakar, C. Langner, I. Nagtegaal, G. Puppa, R. Riddell, A. Ristimaki, K. Sheahan, T. Smyrk, K. Sugihara, B. Terris, H. Ueno, M. Vieth, I. Zlobec, P. Quirke. Recommendations for reporting tumour budding in colorectal cancer based on the international tumour budding consensus conference (itbcc) *Eur Congress Pathol*. Köln: Virchows Arch, 2016.
15. Sobin LH, Gospodarowicz MK, Wittekind C, International Union against Cancer. *Tnm classification of malignant tumours*. 7th ed. Chichester, West Sussex, UK ; Hoboken, NJ: Wiley-Blackwell, 2010;xx, 310 p.
16. Domingo E, Ramamoorthy R, Oukrif D *et al.* Use of multivariate analysis to suggest a new molecular classification of colorectal cancer. *The Journal of pathology* 2013;**229**;441-448.
17. Dorta G, Mottet C. [revised consensus guidelines of the swiss society of gastroenterology: Follow up after colonoscopic polyp removal and colorectal cancer] *Swiss Medical Forum* 2016;**16**;164-167.
18. Rogers AC, Winter DC, Heeney A *et al.* Systematic review and meta-analysis of the impact of tumour budding in colorectal cancer. *British journal of cancer* 2016;**115**;831-840.
19. Karamitopoulou E, Zlobec I, Kolzer V *et al.* Proposal for a 10-high-power-fields scoring method for the assessment of tumor budding in colorectal cancer. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 2013;**26**;295-301.

20. Zlobec I, Hadrich M, Dawson H *et al.* Intratumoural budding (itb) in preoperative biopsies predicts the presence of lymph node and distant metastases in colon and rectal cancer patients. *British journal of cancer* 2014;**110**;1008-1013.
21. Ueno H, Mochizuki H, Hashiguchi Y *et al.* Risk factors for an adverse outcome in early invasive colorectal carcinoma. *Gastroenterology* 2004;**127**;385-394.
22. Lugli A, Karamitopoulou E, Panayiotides I *et al.* Cd8+ lymphocytes/ tumour-budding index: An independent prognostic factor representing a 'pro-/anti-tumour' approach to tumour host interaction in colorectal cancer. *British journal of cancer* 2009;**101**;1382-1392.
23. Horcic M, Koelzer VH, Karamitopoulou E *et al.* Tumor budding score based on 10 high-power fields is a promising basis for a standardized prognostic scoring system in stage ii colorectal cancer. *Human pathology* 2013;**44**;697-705.
24. Koelzer VH, Zlobec I, Berger MD *et al.* Tumor budding in colorectal cancer revisited: Results of a multicenter interobserver study. *Virchows Archiv : an international journal of pathology* 2015;**466**;485-493.
25. Ordonez NG. Broad-spectrum immunohistochemical epithelial markers: A review. *Human pathology* 2013;**44**;1195-1215.
26. Gurzu S, Jung I. Aberrant pattern of the cytokeratin 7/cytokeratin 20 immunophenotype in colorectal adenocarcinomas with braf mutations. *Pathology, research and practice* 2012;**208**;163-166.
27. Gurzu S, Serester O, Jung I. Possible neuroendocrine phenotype of poorly differentiated cell clusters in colorectal carcinoma, as a prognostic parameter. *The American journal of surgical pathology* 2014;**38**;143-144.

FIGURE LEGENDS

Figure 1: Study Design.

Figure 2: Scoring method for topographic assessment of tumour budding showing peritumoral (PTB), intratumoral (ITB) and overall tumor budding (OTB) evaluation.

Table 1: Descriptive statistics for PTB, ITB and OTB (n=215)

Budding	PTB_{10HPF}	ITB_{10HPF}	OTB_{10HPF}	$PTB_{hotspot}$	$ITB_{hotspot}$	$OTB_{hotspot}$
Mean	8.5	7.6	8.0	13.8	11.8	15.8
Median	6	4.8	5.5	10	8.0	12
Min	0	0	0	0	0	0
Max	85	67	64.5	96	82	96

Table 2: Correlation coefficients (r) underling the linear relationships between PTB, ITB and OTB (n=215)

	PTB_{10HPF}	ITB_{10HPF}	OTB_{10HPF}	$PTB_{hotspot}$	$ITB_{hotspot}$	$OTB_{hotspot}$
PTB_{10HPF}	1.0					
ITB_{10HPF}	0.81	1.0				
OTB_{10HPF}	0.95	0.95	1.0			
$PTB_{hotspot}$	0.93	0.74	0.88	1.0		
$ITB_{hotspot}$	0.83	0.93	0.93	0.77	1.0	
$OTB_{hotspot}$	0.91	0.85	0.94	0.94	0.91	1.0
*All correlations $p<0.0001$						

Table 3: Association of PTB, ITB and OTB scores with clinicopathological features by continuous and cut-off scores (n=215; p-values are shown)

	Evaluation by continuous scores						Evaluation by cut-off scores					
	PTB_{10HPF}	ITB_{10HPF}	OTB_{10HPF}	$PTB_{hotspot}$	$ITB_{hotspot}$	$OTB_{hotspot}$	PTB_{10HPF}	ITB_{10HPF}	OTB_{10HPF}	$PTB_{hotspot}$	$ITB_{hotspot}$	$OTB_{hotspot}$
Gender	0.1804	0.1615	0.1224	0.2413	0.2871	0.0953	0.1025	0.064	0.0798	0.4492	0.8238	0.0879
Histology	**0.0006**	**0.0025**	**0.0011**	**0.0013**	**0.0019**	0.1137	**0.0017**	0.0953	**0.011**	**0.0172**	0.0629	0.1769
Location	0.0744	0.1248	0.0652	0.1564	**0.05**	**0.04**	**0.0438**	0.4003	0.0545	0.1411	0.5509	0.0607
pT	**0.0004**	**<0.0001**	**<0.0001**	**0.0058**	**0.0015**	**0.0022**	**0.0354**	**0.0017**	**0.0028**	**0.0357**	**0.0001**	0.0837
pN	**0.0004**	**<0.0001**	**<0.0001**	**0.0015**	**0.0001**	**0.0002**	**0.0034**	**0.0001**	**0.0009**	**0.0127**	**0.0004**	**0.0011**
cM	**0.0117**	**<0.0001**	**0.0006**	0.2554	**0.0006**	**0.0414**	0.2131	**0.0004**	**0.0028**	0.6364	**0.0008**	0.1932
L	**<0.0001**	**<0.0001**	**<0.0001**	**0.0001**	**0.0004**	**<0.0001**	0.082	**<0.0001**	**0.0013**	**0.0001**	**0.0001**	**0.0044**
V	**0.0013**	**0.0015**	**0.0005**	**0.0029**	**0.0136**	**0.0019**	0.2273	0.192	**0.0041**	**0.0011**	0.1456	**0.0048**
Pn	**0.0104**	**0.0003**	**0.002**	**0.0197**	**0.0107**	**0.0234**	0.155	**0.0219**	**0.0251**	0.0846	**0.0393**	0.1162
G	**0.0115**	**0.0002**	**0.0012**	**0.0304**	**0.001**	**0.0046**	**0.0024**	**0.0118**	**0.0017**	0.2442	**0.0462**	0.1084
TBC	**<0.0001**	**0.0002**	**0.0063**	**<0.0001**	**0.0013**	**0.0003**	**0.0291**	**<0.0001**	**0.0016**	**0.0278**	**0.0005**	**0.0179**
Post TX	**0.0006**	**<0.0001**	**0.0001**	**0.0011**	**0.0004**	**0.0002**	**0.0009**	**0.0002**	**0.0007**	0.0743	**0.0059**	0.201
MSI	0.6399	0.1533	0.3068	0.9253	0.1577	0.4818	0.4427	0.6337	0.8336	0.7724	0.6003	0.9385
DFS	**0.0315**	0.0632	**0.0309**	0.1112	**0.0126**	**0.05**	0.6681	0.7198	0.7831	0.8864	0.5243	0.4534
HR (95%CI)	1.028 (1.002-1.053)	1.029 (0.998-1.06)	1.032 (1.003-1.062)	1.018 (0.996-1.041)	1.028 (1.006-1.051)	1.021 (1.000-1.042)						

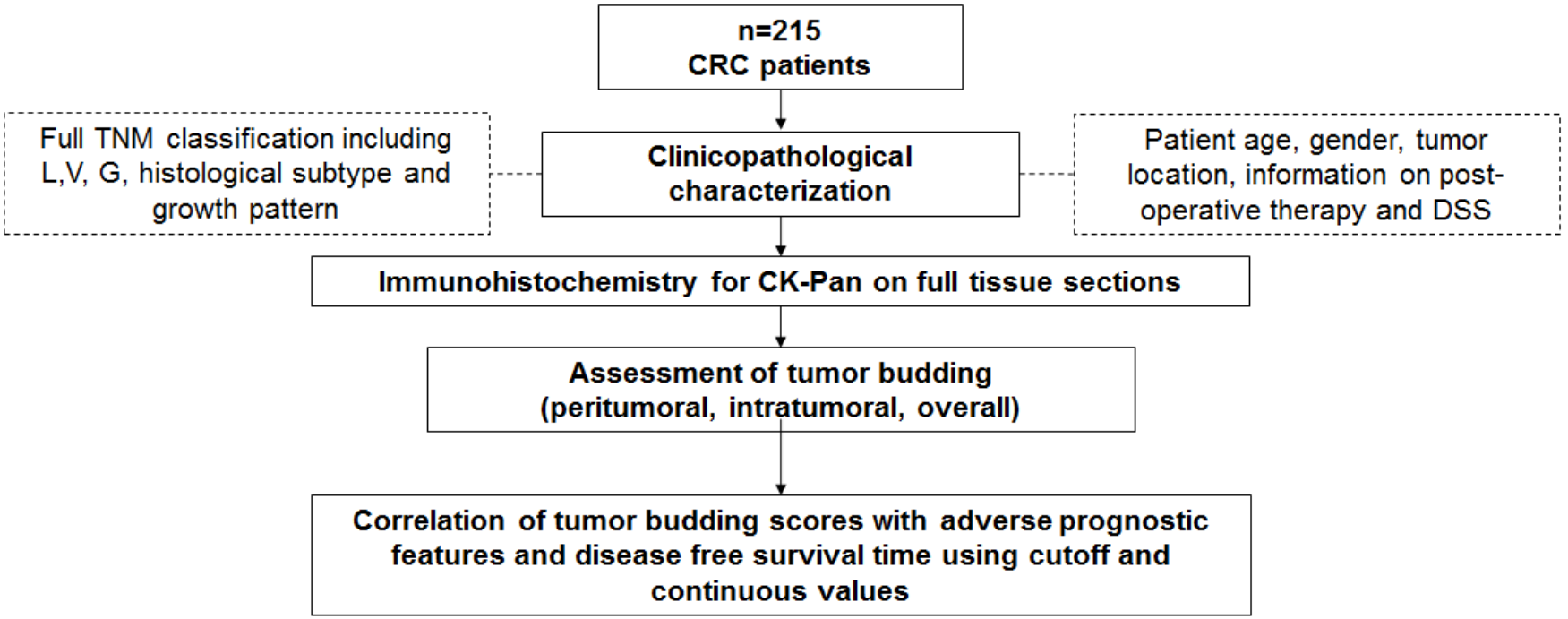

Figure 1

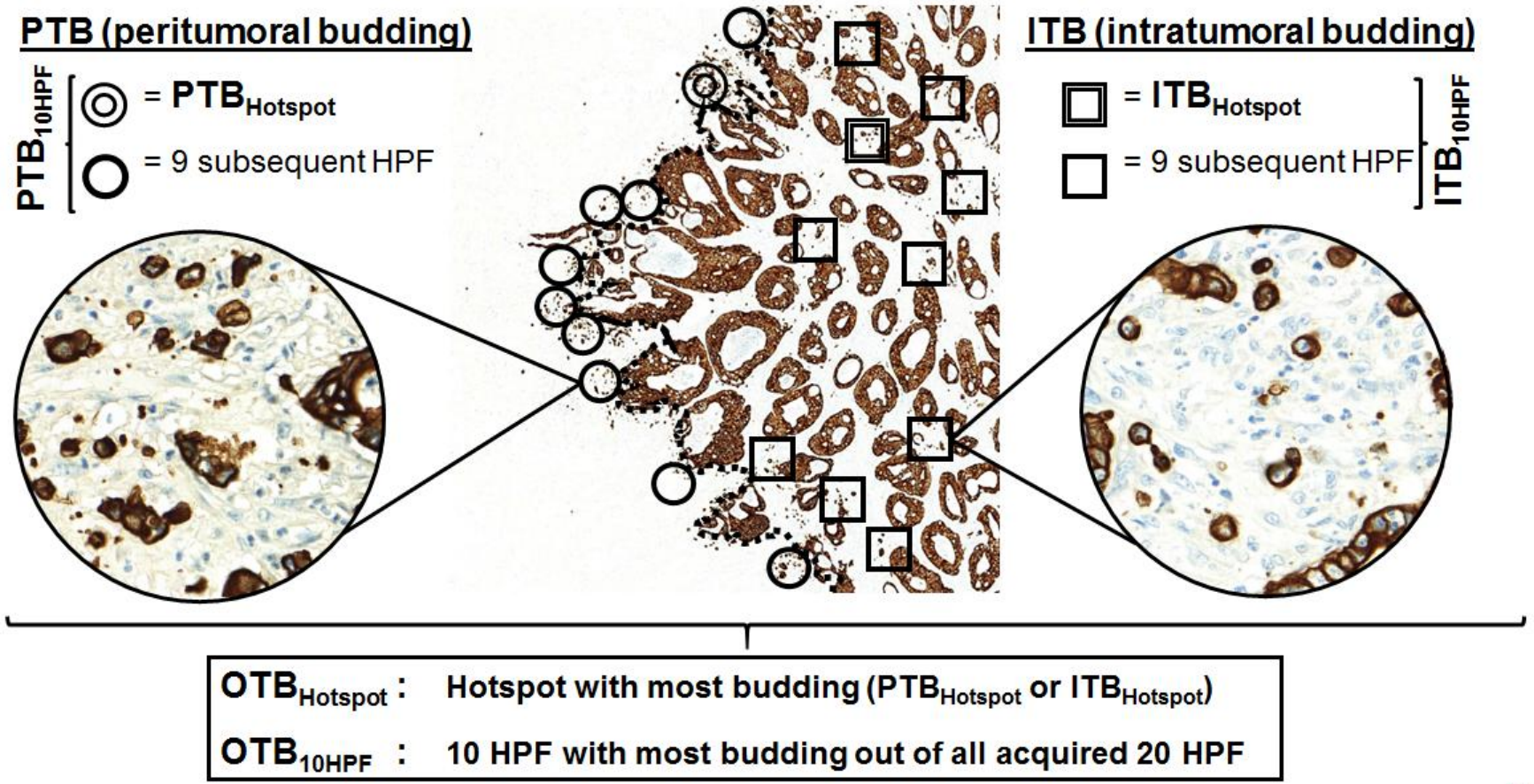

Figure 2